Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals

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We recently found that long-term exposure to nitric oxide (NO) triggers mitochondrial biogenesis in mammalian cells and tissues by activation of guanylate cyclase and generation of cGMP. Here, we report that the NO/cGMP-dependent mitochondrial biogenesis is associated with enhanced coupled respiration and content of ATP in U937, L6, and PC12 cells. The observed increase in ATP content depended entirely on oxidative phosphorylation, because ATP formation by glycolysis was unchanged. Brain, kidney, liver, heart, and gastrocnemius muscle from endothelial NO synthase null mutant mice displayed markedly reduced mitochondrial content associated with significantly lower oxygen consumption and ATP content. In these tissues, ultrastructural analyses revealed significantly smaller mitochondria. Furthermore, a significant reduction in the number of mitochondria was observed in the subsarcolemmal region of the gastrocnemius muscle. We conclude that NO/cGMP stimulates mitochondrial biogenesis, both *in vitro* and *in vivo*, and that this stimulation is associated with increased mitochondrial function, resulting in enhanced formation of ATP.

ATP | cGMP | oxygen consumption

Nitric oxide (NO) is a ubiquitous signaling molecule involved in various physiological functions. It can also mediate deleterious effects that become apparent after its inappropriate or excessive formation (1, 2). Some of the physiological and pathological effects of NO result from its actions at the mitochondrial level. At nanomolar concentrations, NO binds to cytochrome *c* (Cyt *c*) oxidase (complex IV, COX-IV), the terminal enzyme in the mitochondrial electron-transport chain, inhibiting its activity reversibly and in competition with O_2 (3–5). NO-dependent regulation of mitochondrial respiration and membrane potential contributes to acute O_2 sensing by the cells (6–9). Furthermore, binding of NO to COX-IV leads to a switch to glycolysis in competent cells, redistribution of O_2, and regulation of the levels of the hypoxia-inducible factor 1 α, thus contributing to long-term adaptation to hypoxic conditions (10, 11). By contrast, high concentrations of NO persistently inhibit complexes I and II of the respiratory chain, as well as enzymes of the glycolytic pathway and Krebs cycle, thus leading to metabolic imbalance and cell damage (12–15).

We have recently shown (16) that long-term exposure of cells in culture to low concentrations of NO induces mitochondrial biogenesis. This process is mediated by cGMP, resulting from NO-dependent activation of ''soluble'' guanylate cyclase (sGC), and involves increased expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (Tfam). Other mitochondrial biogenetic stimuli also increase the expression of these transcription factors (17–20). NO-dependent mitochondrial biogenesis is important for cell and tissue metabolism, as demonstrated by studies in mice deficient in endothelial NO synthase (eNOS). Reduced mitochondrial biogenesis in tissues of these animals is associated with reduced energy expenditure and increased body weight (16, 21). In this study, we have characterized the functional state of mitochondria generated by the action of NO and demonstrated that NO/cGMP-dependent mitochondrial biogenesis yields functionally active mitochondria, in terms of respiratory function and metabolic activity, in various mammalian cells as well as in animal tissues.

Materials and Methods

Materials. The following reagents were purchased as indicated: (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2 diolate (DETA-NO), and *H*-(1,2,4)-oxadiazolo[4,3-]quinoxalin-1-one (ODQ) from Alexis Italia (Florence, Italy); BAY 41-2272 from Bayer (Wuppertal, Germany); primary mAbs anti-subunit IV of COX-IV from Molecular Probes; anti-Cyt *c* mAbs from Pharmingen; and fluorescein isothiocyanate-labeled goat-anti-rabbit IgG from The Jackson Laboratory. Fetal clone III was obtained from HyClone–Celbio (Milan). All other cell culture reagents were obtained from GIBCO (Basel). Carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) and all other reagents were obtained from Sigma–Aldrich (Milan).

Animals and Tissues. Male 8- to 10-week-old WT and eNOS null mutant ($eNOS^{-/-}$) mice (16) were housed in the Pathogen-Free Facility in the Department of Preclinical Sciences at the University of Milan. Animals were treated in accordance with European Community guidelines and with the approval of the Institutional Ethical Committee. On the day of the experiments, animals were killed by cervical dislocation, and tissues were isolated immediately. They were either frozen in liquid nitrogen (for mtDNA, mitochondrial protein, and ATP studies), or they were freed of connective tissue, fat, and large vasculature; cut into 20- to 30-mg slices; and maintained in a Krebs' solution containing 118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, and 10 mM glucose (pH 7.4) in which $20\%O_2/5\%CO_2/75\%N_2$ was bubbled continuously at 37°C (for the O_2-consumption experiments).

Cell Culture and Treatments. U937, L6, and PC12 cells were cultured essentially as described (22–24). At day 0, U937 cells were suspended at a density of 3×10^4 cells per ml, whereas L6

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Abbreviations: sGC, ''soluble'' guanylate cyclase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; NRF-1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor A; eNOS, endothelial NO synthase; DETA-NO, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2 diolate; ODQ, *H*-(1,2,4)-oxadiazolo[4,3-]quinoxalin-1-one; Cyt *c*, cytochrome *c*; FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone.

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and PC12 were seeded at a density of 1×10^5 cells per mm^2. DETA-NO, 8 Br-cGMP, BAY 41-2272, and ODQ were added to the cultures in various combinations, as indicated in *Results*, one time per day for 6 days. After this time, to minimize the possible effects of nutrient deprivation on the cells, they were washed once in drug-free, fresh culture medium and equilibrated in it for an additional 3 h at 37°C. Cells were then collected and washed twice in PBS. For the O_2-consumption and flow-cytometry experiments, the cells were suspended (10^7 cells per ml) in a buffer containing 118 mM NaCl, 4.8 mM KCl, 1.2 KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 25 mM Hepes (pH 7.2). For the remaining experimental procedures, the cells were sedimented by centrifugation and kept at −80°C until use.

Analysis of mtDNA and Proteins. Mitochondria were isolated from cultured cells and tissues as described (25). Extraction and purification of mtDNA from lysed mitochondria was carried out as described (16). Aliquots of mtDNA was loaded on ethidium bromide-stained agarose gel (1.2%) and analyzed by using the QuickImage densitometer (Packard). The mtDNA levels were normalized to the protein content, which was measured by using the bicinchoninic acid protein-assay procedure (Perbio, Bezons, France). Analysis of the cellular content of mitochondrial proteins was carried out by flow cytometry, as described (26). Briefly, cell suspensions were permeabilized for 20 min at room temperature in the presence of 1% BSA and 0.1% saponin. Samples (1×10^6 cells) were then incubated with the appropriate primary Abs for 30 min at 4°C. Expression of these molecules was analyzed by flow cytometry after staining with appropriate fluorescein isothyocyanate-labeled Abs, by using a fluorescence-activated cell sorter (FACStar Plus, Beckton Dickinson) (26).

Quantitative RT-PCR. Quantitative RT-PCR was carried out by using an ABI Prism 7700 sequence-detection system and TaqMan (Applied Biosystems), which uses the 5′ nuclease activity of TaqDNA polymerase to generate a real-time quantitative DNA-analysis assay (16). Briefly, gene-specific oligonucleotide probes with 5′ fluorescent and 3′ rhodamine (quench) moieties were designed and used for the extension phase of the PCR. The degradation and release of the fluorescent moiety results in fluorescence at 518 nm, which is monitored during the complete amplification process. Comparisons with glyceraldehyde-3-phosphate dehydrogenase (internal control) and individual standard curves were carried out in parallel.

Measurement of O_2 Consumption. We analyzed 1-ml cell or tissue samples at 37°C in a gas-tight vessel that was equipped with a Clark-type O_2 electrode (Rank Brothers, Bottisham, U.K.) connected to a chart recorder. Cellular O_2 consumption was measured as described (14). The O_2 electrode was calibrated by assuming the concentration of O_2 in the incubation medium at 37°C to be 200 μM. The uncoupler FCCP was added directly to the cuvette, whereas oligomycin was added 20 min before O_2-consumption measurements. Protein content in both cell and tissue samples was determined by the bicinchoninic acid protein assay.

Measurements of ATP, Lactate, and Glycolytic Enzyme Activities. The ATP content of cultured cells and animal tissues was determined in 2.5% perchloric acid extracts neutralized with K_2CO_3, by reversed-phase HPLC, as described (27). Lactate was measured in the culture medium after deproteinization by using Ultra-4 centrifugal filter devices (cut-off, 10,000; Amicon) as well as in cells after extraction in cold perchloric acid as described (28). The activities of the enzymes hexokinase (EC 2.7.1.1) and glyceraldehyde phosphate dehydrogenase (EC 1.2.1.13) were determined in cell lysates obtained by sonication (three pulses of 30 sec at 100 W) as described (28).

Electron Microscopy. Liver and gastrocnemius muscle were carefully removed from WT and $eNOS^{-/-}$ mice, cut into pieces of ≈1 mm^3, and placed in ice-cold fixative (2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 5 h. Samples were then washed extensively with 0.1 M cacodylate buffer, postfixed for 2 h with 2% OsO_4/0.1 M cacodylate buffer, dehydrated in ethanol, block-stained with uranyl acetate, and embedded in Epon. Ultrathin sections were collected on copper grids, doubly stained with uranyl acetate and lead citrate, and examined under a CM10 transmission electron microscope (Philips, Eindhoven, the Netherlands) (23). For morphometric studies of mitochondria, randomly selected areas of tissue derived from three animals per group were photographed at a ×11,500 magnification and analyzed with National Institutes of Health IMAGE software. Statistical analyses of cross-sectional area of mitochondria and mitochondrial densities were carried out by using PRISM 2.0 software.

Statistical Analysis. The results are expressed as means ± SEM; n represents the number of individual experiments. Statistical analysis was performed by using Student's t test for unpaired variables (two-tailed). Single, double, and triple asterisks in the tables and figures indicate statistical probabilities of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Results

Human monocytic U937 cells, rat L6 myoblasts, and rat PC12 neurosecretory cells were treated every day for 6 days with various combinations of the following agents: the NO donor DETA-NO [50 μM; yielding a NO concentration of 98 ± 9.2 nM ($n = 3$), as measured by an NO electrode (14)], the membrane-permeant cGMP analogue 8 Br-cGMP (3 mM), BAY 41-2272 [1 μM; a compound that activates sGC through an NO-independent site (29)], and the sGC inhibitor ODQ (1 μM). None of the treatments affected cell viability, as assessed daily by the trypan blue exclusion assay (data not shown).

Treatment for 6 days with DETA-NO enhanced the expression of PGC-1α, NRF-1, and Tfam, albeit to different degrees, in the three cell types (Fig. 1). Similarly, mtDNA content was increased, as was the expression of the mitochondrial proteins COX-IV and Cyt c (Fig. 2). The effects of DETA-NO were mimicked by either 8 Br-cGMP or BAY 41-2272 and were prevented by ODQ (Fig. 1 and 2). Thus, activation of sGC and the ensuing generation of cGMP appear to be both necessary and sufficient to promote NO-dependent mitochondrial biogenesis in three different types of cells in culture.

Next, the impact of the NO-dependent mitochondrial biogenesis on cellular respiration was investigated. In view of the cGMP dependency of the process, we used cells treated for 6 days with either 8 Br-cGMP or BAY 41-2272, which are compounds that share with NO an effect on the cGMP system without sharing its effects on COX-IV or on cGMP-independent signaling pathways (1–6). These treatments modified cell protein content differently in the investigated cell lines. There were net increases of 25 ± 1.23% and 20 ± 1.21% in U937 cells and 12.5 ± 0.65% and 5.7 ± 0.11% in PC12 cells after 8-Br cGMP and BAY 41-2272, respectively ($n = 3$), whereas no changes in net protein content were observed in the L6 cells. Therefore, results were normalized to protein content to allow better comparison of the effects on mitochondrial biogenesis. Consumption of O_2 was measured under basal conditions and after the addition of FCCP (2 μM) or oligomycin (2.5 μM). FCCP completely uncouples mitochondria and maximizes their respiratory capacity, thus reflecting the maximal electron transport activity of mitochondria. In contrast, the F1/F0 ATP synthetase inhibitor oligomycin blocks oxidative phosphorylation-linked O_2 consumption, without affecting O_2 consumption occurring through proton leakage. Comparison of the O_2 consumption values under the three experimental con-

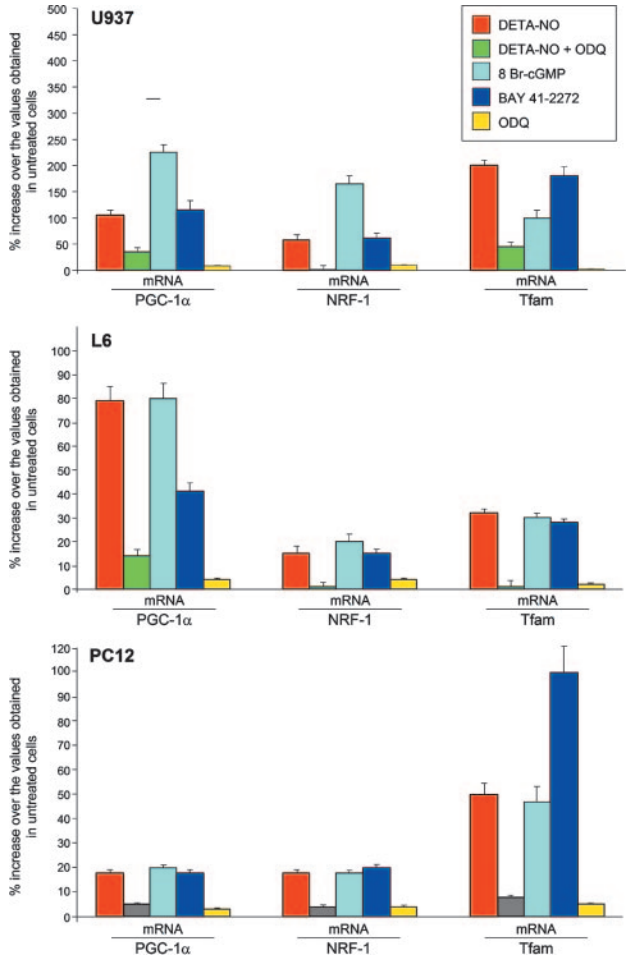

Fig. 1. NO triggers transcription of genes involved in mitochondrial biogenesis in different mammalian cells through cGMP generation. U937, L6, and PC12 cells were cultured for 6 days with or without 50 μM DETA-NO, 3 mM 8 Br-cGMP, 1 μM BAY 41-2272, or 1 μM ODQ, as indicated. PGC-1α, NRF-1, and Tfam mRNA levels were analyzed by means of quantitative RT-PCR analysis with gene-specific oligonucleotide probes. Specific fluorescence was monitored during the complete amplification process and compared with glyceraldehyde-3-phosphate dehydrogenase (internal control) fluorescence. mRNAs levels were expressed as percentages of increase over the values obtained in untreated cells ($n = 5$). With all treatments, except those with DETA-NO plus ODQ or ODQ alone, the percentages of increase over basal values were statistically significant ($P < 0.01$).

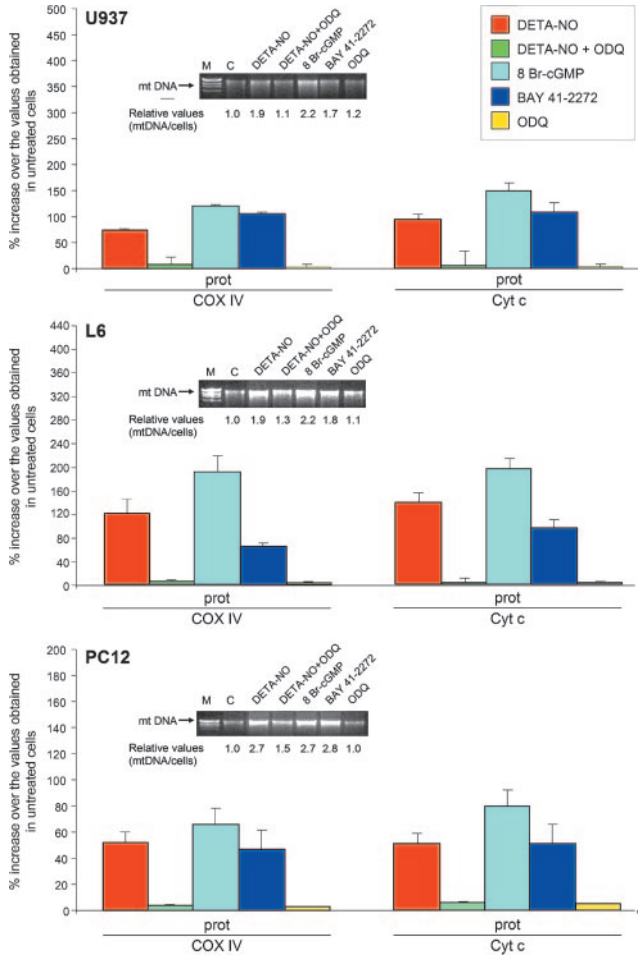

Fig. 2. NO increases mitochondrial content in different mammalian cells through cGMP generation. U937, L6, and PC12 cells were cultured for 6 days with or without 50 μM DETA-NO, 3 mM 8 Br-cGMP, 1 μM BAY 41-2272, or 1 μM ODQ, as indicated. The following parameters were then evaluated: mtDNA (one experiment representative of five; the numbers below the gels show the relative values from the densitometric analysis when control measurements are given a value of 1.0), COX-IV, and Cyt *c* proteins (prot), detected by immunoblot analysis. Protein levels were expressed as percentages of increase over the values obtained in untreated cells ($n = 5$). With all treatments, except those with DETA-NO plus ODQ or ODQ alone, the percentages of increase over basal values were statistically significant ($P < 0.01$).

ditions allows an estimate of coupled and uncoupled respiration (19). Treatment with either 8 Br-cGMP or BAY 41-2272 increased the basal, FCCP-stimulated and oligomycin-resistant O_2 consumption rates in the three cell types (Fig. 3). The increases in O_2 consumption rates induced by each compound under basal conditions were similar in each cell type to those measured in the presence of FCCP and oligomycin. This finding shows that both 8 Br-cGMP and BAY 41-2272 increased coupled respiration, regardless of whether or not mitochondrial biogenesis was associated with changes in protein content.

Next, we investigated whether the increase in coupled respiration was associated with an increase in ATP synthesis, and we found that treatment with 8 Br-cGMP or BAY 41-2272 increased cellular ATP levels in the three cell types significantly (Fig. 4*A*). ATP levels depend not only on mitochondrial content but also on ADP availability and changes in energy demand. Notably, these parameters are modified significantly during cell division. Although 8 Br-cGMP and BAY 41-2272 did not modify proliferation of U937 cells, they inhibited proliferation of L6 cells by 34.0 ± 2.11% and 18.1 ± 0.83%, and of PC12 by 92.5 ± 5.41% and 80.5 ± 4.31%, respectively ($n = 3$). The observation that ATP levels increased regardless of the effects on cell proliferation rules out the possibility that ATP accumulates because of the lower utilization due to reduced proliferation.

Immortalized cells in culture normally meet their energy requirements from glycolysis, accounting for the formation of ≈90% of total ATP (30). Thus, short-term exposure to a respiratory chain inhibitor should provide only minor effects in cells in which the ATP pool depends mainly on glycolysis, whereas much greater effects should occur under conditions in which the contribution of mitochondrially generated ATP becomes more significant. We found that exposure of untreated U937 cells to the complex I inhibitor rotenone (1 μM for 60 min) suppressed O_2 consumption (data not shown) but decreased cellular ATP content only marginally (by 12.7%; Fig. 4*B*). However, rotenone caused a much larger decrease in ATP levels in cells that had been treated for 6 days with either

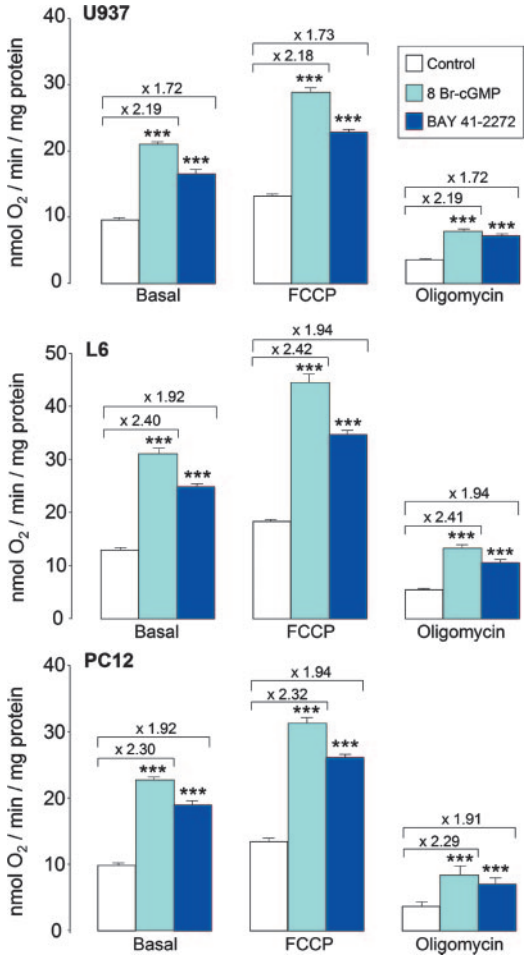

Fig. 3. cGMP-dependent mitochondrial biogenesis leads to an increase in oxidative phosphorylation-coupled respiration. U937, L6, and PC12 cells were cultured for 6 days with or without 3 mM 8 Br-cGMP or 1 μM BAY 41-2272, as indicated. O_2 consumption was measured in a gas-tight chamber by using an O_2 electrode (as described in *Materials and Methods* in samples; 10^7 cells per sample) from the various cell preparations in the presence or absence of the mitochondrial uncoupler FCCP (2 μM) or the F1/F0 ATP synthetase inhibitor oligomycin (2.5 μM). O_2 consumption values were normalized to the protein content of the cells ($n = 6$). In each image, the numbers above the bars indicate the fold increase in respiration over basal value induced by cell treatment with 8 Br-cGMP or BAY 41-2272. Asterisks indicate statistical significance, as described in *Materials and Methods*, vs. controls.

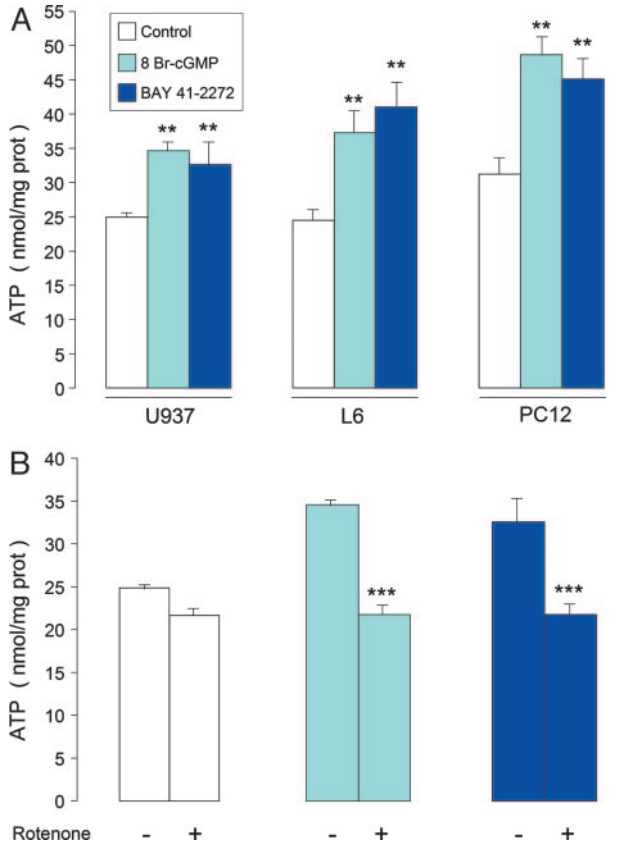

Fig. 4. cGMP-dependent mitochondrial biogenesis leads to increases in steady-state ATP levels through oxidative phosphorylation. U937, L6, and PC12 cells were cultured for 6 days with or without 3 mM 8 Br-cGMP or 1 μM BAY 41-2272, as indicated. (*A*) Cellular steady-state ATP levels in the various cell preparations. (*B*) Steady-state ATP levels, measured in U937 cells in the presence or absence of the complex I inhibitor rotenone (1 μM). Asterisks indicate statistical significance, as described in *Materials and Methods*, vs. controls ($n = 3$).

8 Br-cGMP or BAY 41-2272, so that they were the same as those detected in untreated cells (Fig. 4*B*). Treatment of each cell type with deoxyglucose (5 mM) for 60 min resulted in a marked fall in ATP content, so that similar amounts remained in control cells and in those treated with 8 Br-cGMP or BAY 41-2272 (data not shown). These results strongly suggest that the cGMP-mediated increase in the ATP content can be attributed to oxidative phosphorylation.

To confirm these observations, lactate concentrations in the extracellular/intracellular milieu were measured to obtain an estimate of the glycolytic flux, in conditions identical to those used for the ATP determination. Lactate concentrations were similar in the different treatment conditions both in the extracellular milieu (values were 3.82 ± 0.27 mM and 3.91 ± 0.25 mM in untreated and 8 Br-cGMP-treated cells, respectively; $n = 4$) and within the cells (values were 0.098 ± 0.015 μmol/mg protein and 0.099 ± 0.013 μmol/mg protein in untreated and 8 Br-cGMP-treated cells, respectively; $n = 4$). Furthermore, the activity of two key enzymes of glycolysis [namely, hexokinase (0.104 ± 0.019 units/mg protein and 0.0985 ± 0.016 units/mg protein in untreated and 8 Br-cGMP-treated cells, respectively; $n = 4$) and glyceraldehyde phosphate dehydrogenase (1.005 ± 0.021 units/mg protein and 1.025 ± 0.14 units/mg protein in untreated and 8 Br-cGMP-treated cells, respectively; $n = 4$)] did not show appreciable variations. Results similar to those observed in U937 cells were obtained in L6 and PC12 cells (data not shown). Thus, mitochondrial biogenesis triggered by cGMP results in functionally active mitochondria, so that cells consume more O_2 and generate more ATP by a process independent of glycolysis.

Previous studies in eNOS$^{-/-}$ mice have indicated that NO regulates mitochondrial biogenesis *in vivo* (16, 21). Therefore, we have investigated the relationship among mitochondrial biogenesis, O_2 consumption, and generation of ATP in the brain, heart, liver, kidney, and gastrocnemius muscle from WT and eNOS$^{-/-}$ mice. Basal mitochondrial content (assessed by measuring mtDNA, basal O_2-consumption rates, and ATP content) was reduced in tissues from eNOS$^{-/-}$ mice when compared with their WT counterparts (see Table 2, which is published as supporting information on the PNAS web site). These results indicate that NO generation by eNOS *in vivo* is required to maintain functionally active mitochondria able to couple increased O_2 consumption with generation of ATP. The lower mitochondrial content in eNOS$^{-/-}$ mice was accompanied in all

Table 1. Morphometric analyses of mitochondria in tissues from WT and eNOS$^{-/-}$ mice

	Liver (total)		Intramyofibrillar gastrocnemius		Subsarcolemmal gastrocnemius	
Analysis	WT	eNOS$^{-/-}$	WT	eNOS$^{-/-}$	WT	eNOS$^{-/-}$
Mean area	0.66 ± 0.025	0.52 ± 0.019**	0.19 ± 0.004	0.16 ± 0.004***	0.23 ± 0.010	0.21 ± 0.004*
Density	0.39 ± 0.011	0.36 ± 0.012*	0.84 ± 0.001	0.70 ± 0.003**	2.0 ± 0.010	0.83 ± 0.003***

Morphometric analyses were carried out on micrographs at the electron microscopy level by using National Institutes of Health IMAGE and PRISM 2.0 software as described in *Materials and Methods*. Mean area values (μm^2 per mitochondrion) ± SEM were calculated in 1,000 mitochondria from each tissue preparation. The mitochondrial density was calculated taking into account 50 micrographs from each tissue preparation. Density is expressed either as number of mitochondria per μm^2 of cytoplasm (liver and intramyofibrillar pool of the gastrocnemius muscle) or mitochondria per μm of sarcolemma (subsarcolemmal pool of the gastrocnemius mucle). Asterisks indicate statistical significance, as described in *Materials and Methods,* vs. WT.

tissues by lower PGC-1α mRNA levels, compatible with the involvement of PGC-1α expression in mitochondrial biogenesis in tissues as in cells (Table 2).

Last, we compared WT and eNOS$^{-/-}$ mice by morphometric analysis of mitochondria at the ultrastructural level. The results obtained on two representative tissues (liver and gastrocnemius) are reported in Table 1, and representative micrographs are shown in Fig. 5, which is published as supporting information on the PNAS web site. In the hepatocytes from WT animals, mitochondria were packed uniformly throughout the cytoplasm and were round in shape with regular disposition of cristae. In the hepatocytes from eNOS$^{-/-}$ mice, mitochondria were significantly smaller and less densely packed. Two clearly distinct mitochondrial pools were present in muscle fibers (one intramyofibrillar and one subsarcolemmal). Morphometric analysis of the intramyofibrillar pool gave results similar to those observed in the liver, because mitochondria from eNOS$^{-/-}$ tissues were significantly smaller and less densely packed. This finding was accompanied by a marked decrease in the number of mitochondria residing in the subsarcolemmal region (Fig. 5).

Discussion

Our present results show that mitochondrial biogenesis, as assessed by measurements of mtDNA, COX-IV, and Cyt *c* expression, was induced similarly by DETA-NO in three cell types from different species and lineages (i.e., human monocytic U937, rat L6 myoblasts, and neurosecretory PC12 cells). Mitochondrial biogenesis was accompanied by increased expression of PGC-1α, NRF-1, and Tfam (although to variable degrees in the different cell types), and it was mediated by cGMP because 8 Br-cGMP mimicked, and the sGC inhibitor ODQ abolished, the effects of DETA-NO. The observation that BAY 41-2272 triggered mitochondrial biogenesis by means of the same signaling pathway activated by NO confirmed the key role of cGMP in this process. It was further established that endogenous NO plays a critical role in mitochondrial biogenesis (16, 21, 31) in animal models because the mitochondrial content of various tissues from eNOS$^{-/-}$ mice was markedly lower than that of WT animals. These results, together with the results reported in ref. 16, clearly show that mitochondrial biogenesis is a general phenomenon that occurs in cells in culture and animal tissues, most likely through the same cascade of signaling events.

Although NO/cGMP-induced increases in mtDNA content, Cyt *c*, COX-IV PGC-1α, Tfam, and NRF-1 indicate stimulation of mitochondrial biogenesis, these increases do not necessarily imply that the resultant mitochondria are functional. Therefore, we measured two parameters of mitochondrial function (i.e., O_2 consumption and ATP production), and we found that NO/cGMP-induced mitochondrial biogenesis was accompanied by increased O_2 consumption through coupled cellular respiration functionally linked to enhanced ATP production. Interestingly, the cell lines used are highly glycolytic but still respond to cGMP by increasing oxidative phosphorylation. The fact that we did not observe concomitant inhibition in the rate of ATP production by glycolysis may be attributable to the fact that such control of glycolysis is low (32) in the highly glycolytic tumor-derived cells that we used.

cGMP is known to regulate various intracellular signaling pathways involved in key biological processes, including cell proliferation and differentiation (1, 2). Therefore, it is not surprising that, in addition to mitochondrial biogenesis, we observed NO/cGMP-induced changes in cell protein content and/or proliferation rates. However, unlike the consistent changes in O_2 consumption and ATP content, changes in cell protein content and/or proliferation rates were variable and significantly smaller in the three investigated cell types. These results strongly suggest that the increases in O_2 consumption and ATP content are not simply the consequence of changes in cellular protein content and/or proliferation rates.

The functional characterization of mitochondria in animal tissues confirmed and extended the results that we obtained with the cells in culture. The reduced mitochondrial content in tissues from eNOS$^{-/-}$ mice was accompanied by reduction of both basal O_2 consumption and steady-state ATP levels, associated with a significant reduction in the mitochondrial size. These observations occurred in tissues that depend on oxidative metabolism (such as the brain) and in glycolytic tissues (such as the gastrocnemius muscle), indicating that the effect of NO on mitochondrial biogenesis is a general phenomenon. Interestingly, in the gastrocnemius muscle, mitochondrial biogenesis has been shown to be important in the conversion of type II (glycolytic) fibers into type I (oxidative) fibers (33).

There is a high degree of flexibility in mitochondrial size, number, and mass, which are mediated by a complex, tightly coordinated network of different regulatory pathways (34). The mitochondrial volume and network extension (35) have been shown to correlate positively with the efficiency of the mitochondrial respiratory function (36). Thus, it is likely that the decrease in mitochondrial size observed in the tissues from eNOS$^{-/-}$ mice is a morphological correlate of the reduced mitochondrial function. The subsarcolemmal pool of the gastrocnemius muscle appears to be particularly dynamic in adapting to chronic changes at the level of contractile activity (37, 38), and it appears to contain mostly mitochondria at an earlier stage of biogenesis (39). These findings correlate well with our observation that this pool of mitochondria is more affected by NO deprivation than intramyofibrillar mitochondria.

Our results obtained from eNOS$^{-/-}$ mice under resting conditions demonstrate that endogenous NO generation by eNOS is required for *in vivo* maintenance of the basal mitochondrial content. This consideration does not rule out the possibility that other NOS isoforms might participate to mitochondrial biogenesis when this process needs to be intensified. For example, the activation of neuronal NOS occurring during skeletal muscle exercise (40) might contribute to the exercise-induced, PGC-1α-dependent mitochondrial biogenesis (33, 41). We also cannot

rule out entirely the possibility that reduction in blood flow secondary to eNOS deficiency (42) contributes indirectly to the observed phenotype. However, this eventuality is unlikely because no major signs of atrophy were observed in the investigated tissues (ref. 21 and data not shown).

Our results may have important consequences in terms of cell and tissue biology, because mitochondrial activity and biogenesis play a critical role in various processes, including the acquisition of brown fat cell features by white adipocytes (43), the switch of skeletal muscle fibers from glycolytic to oxidative metabolism (33), and the regeneration of cardiac and skeletal muscles (20, 44). In addition, impairment of mitochondrial function is associated with neurodegenerative diseases, neuromuscular disorders, liver and heart failure, and type 2 diabetes (20, 45–49). Therefore, the possibility of generating new, metabolically active mitochondria might improve the outcome of these pathologies. In this context, it is interesting that BAY 41-2272 and new NO donor compounds have potential clinical application (50, 51).

In conclusion, our results, combined with previous evidence, indicate that NO is a regulator of cell metabolism through two distinct actions at the mitochondrial level. Thus, the acute O_2 sensing by the cells, regulated by the reversible binding of NO to COX-IV with the ensuing reduction of O_2 consumption (6, 10, 11), appears to be complemented by long-term changes that depend on mitochondrial biogenesis. How these two actions may be coordinated to meet the energy demands of the cell under different conditions remains to be investigated.

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